

Aerosol delivery of virulent *Mycobacterium bovis* to cattle

M.V. Palmer, W. Ray Waters, D.L. Whipple

Bacterial Diseases of Livestock Research Unit, National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, 2300 Dayton Avenue, Ames, IA 50010, USA

Summary *Setting:* Although animal models of aerosol inoculation of *Mycobacterium tuberculosis* and *M. bovis* have been reported using laboratory animals, a model of aerosol delivery of *M. bovis* to cattle has not been reported previously.

Objective: Develop and characterize a model of aerosol delivery of *M. bovis* to cattle, and compare the distribution of lesions in cattle infected with either of two different strains of *M. bovis*, one isolated from cattle (HC2005T), and the other isolated from white-tailed deer (1315).

Design: Cattle ($n=20$, female and castrated males) aged 4 months, were infected with 1×10^3 ($n=5$) or 1×10^5 ($n=5$) colony-forming units (CFU) of *M. bovis* 1315 or 1×10^3 ($n=5$) or 1×10^5 ($n=5$) CFU of *M. bovis* HC2005T. Calves were infected using a commercially available aerosol delivery system. One hundred fifty-five days after infection, calves were euthanized, examined and tissues collected for microscopic analysis and bacteriologic culture.

Results: Nineteen of 20 calves developed tuberculosis. Typical tuberculous lesions were most pronounced in the lungs and tracheobronchial and mediastinal lymph nodes.

Conclusion: The system described provides a reliable method of aerosol delivery of *M. bovis* to cattle. Lesion distribution suggests that the aerosolized inoculum was delivered deep into pulmonary alveoli and thus represents true aerosol exposure. Disease was more severe in groups receiving the highest dose of either inoculum strain; however, differences between strains were not seen. Published by Elsevier Science Ltd.

INTRODUCTION

Mycobacterium bovis is the causative agent of tuberculosis in cattle and other animals, and an important cause of human tuberculosis. Various animal models have been developed to study human and animal tuberculosis.^{1–5} Routes of experimental infection include intravenous, intranasal, intratracheal, intratonsillar, oral, and aerosol. The primary means of transmission of *Mycobacterium tuberculosis* in humans is by aerosol and thus results in pulmonary lesions; however, *M. bovis* infection in humans often results in non-pulmonary lesions, although pulmonary lesions associated with *M. bovis* are also seen.⁶

It is generally accepted that, with some exceptions, cattle become infected with *M. bovis* by either the oral or respiratory routes.⁷ The oral route is most important in calves nursing tuberculous cows, while the respira-

tory route is most common in cattle in general and facilitated by the natural behavior of cattle.⁷ To simulate natural disease, experimental infection of cattle has been done by intranasal,^{8,9} intratracheal,¹⁰ and intratonsillar¹¹ routes. Although aerosol delivery has been used in small animal models of tuberculosis, due to obvious limitations and obstacles, experimental aerosol delivery of *M. bovis* to large animals such as cattle has not been reported.

The objectives of this study were to develop and characterize a model of aerosol delivery of *M. bovis* to cattle, and to compare the distribution of lesions in cattle infected with either of two strains of *M. bovis*, one isolated from cattle, and the other isolated from white-tailed deer (*Odocoileus virginianus*).

MATERIALS AND METHODS

Animals

Twenty, healthy, Maine Anjou cattle (female and castrated male) aged 4 months, were randomly assigned to four groups. Two groups were experimentally infected with

Correspondence to: Mitchell V. Palmer, Bacterial Diseases of Livestock Research Unit, National Animal Disease Center, 2300 Dayton Avenue Ames, IA 5001, USA. Tel.: +1-515-663-7474; fax: +1-515-663-7458; e-mail: mpalmer@nadc.ars.usda.gov

M. bovis strain 1315, originally isolated in 1995 from the medial retropharyngeal lymph node of a hunter-killed white-tailed deer in Michigan, USA. Five animals from this group were infected with 1×10^3 colony-forming units (CFU), and 5 with 1×10^5 CFU of *M. bovis* 1315. Two other groups were experimentally infected with *M. bovis* strain HC2005T, originally isolated from the tracheobronchial lymph node of a dairy cow in Texas, USA with a tuberculin skin test consistent with exposure to *M. bovis*. Five animals from this group were infected with 1×10^3 CFU, and five with 1×10^5 CFU of *M. bovis* HC2005T. Challenge inoculum consisted of mid-log-phase *M. bovis* grown in Middlebrook's 7H9 media supplemented with 10% oleic acid-albumin-dextrose complex (OADC, Difco, Detroit, MI) plus 0.05% Tween 80 (Sigma Chemical Co., St Louis, MO) as described.¹² To harvest tubercle bacilli from the culture media, bacteria were pelleted by centrifugation at 750g, washed twice with phosphate-buffered saline solution (PBS, 0.01 M, pH 7.2), and diluted to the appropriate density for use as inoculum in 2 ml of PBS. Inoculum was sonicated for 5 s prior to nebulization to disperse clumps of bacteria. Enumeration of bacilli was by serial dilution plate counting on Middlebrook's 7H11 selective media (Becton Dickinson, Cockeysville, MD).

M. bovis strains 1315 and HC2005T used in this study have distinct restriction fragment length polymorphism (RFLP) patterns when the polymorphic GC-rich repetitive sequences (PGRS) region is analyzed. Isolates of *M. bovis* obtained from samples of the mediastinal or tracheobronchial lymph nodes of each tuberculous animal at the time of necropsy were analyzed by RFLP, as described previously,¹³ to ensure that the isolate obtained from the animal at necropsy matched that of the isolate used for inoculation.

Experimental infection

For aerosol delivery, cattle were restrained in a head chute and the challenge inoculum delivered by nebulization into a mask covering the nostrils and mouth (Fig. 1). The nebulization apparatus consisted of a compressed air tank and commercially available aerosol delivery system (Equine Aeromask, Trudell Medical, London, Ontario, Canada) comprised of a jet nebulizer (Whisper Jet, Marquest Medical Products, Englewood, CO), holding chamber and mask. According to the manufacturer, in studies using 0.9% saline, the Whisper jet nebulizer produces a particle size with a mass median aerodynamic diameter of $1.91 \mu\text{m}$ with an output of 0.24 ml/min at a flow rate of 8 liters per minute (lpm) and 50 pounds per square inch (psi). A separate study comparing the nebulization of albuterol by various brands of nebulizers, found that at a flow rate of 2 lpm the Whisper jet

nebulizer produced a particle with approximately $5 \mu\text{m}$ diameter.¹⁴ Compressed air (25 psi) was used to jet nebulize inoculum (2 ml *M. bovis* in PBS) directly into the holding chamber. Upon inspiration, the nebulized inoculum was inhaled through a one-way valve into the mask and directly into the nostrils. A rubber gasket sealed the mask securely to the muzzle preventing leakage of inoculum around the mask. Expired air exited through one-way valves on the sides of the mask. The nebulization process continued until all of the inoculum, a 1 ml PBS wash of the inoculum tube, and an additional 2 ml PBS were delivered (approximately 12 min). Experimental infection was done inside a biosafety level 3 (BL-3) building with personnel wearing appropriate personal protective equipment, including full-face respirators with HEPA-filtered canisters to prevent exposure to aerosolized *M. bovis*. The BL-3 animal housing had negative air pressure as compared to the outside. Airflow was such that air was pulled out of animal rooms towards a central corridor, preventing air exchange between rooms. The airflow was adjusted to produce 11.4 air changes per hour. Cattle were housed two per pen (11m^2) according to treatment group in facilities approved by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). A protocol detailing procedures and animal care was approved by the Institutional Animal Care and Use Committee (IACUC) prior to the beginning of the experiment.

Sample collection

One hundred fifty-five days after infection, all cattle were humanely euthanized by intravenous injection of sodium pentobarbital. A thorough postmortem examination was done on each animal and the following tissues collected for bacteriologic isolation of *M. bovis* and microscopic analysis; palatine tonsil; mandibular, parotid, medial retropharyngeal, tracheobronchial, mediastinal, preformal, mesenteric, and hepatic lymph nodes, lung, liver, spleen and nasal turbinate. Tissues were processed for isolation of *M. bovis* as previously described.⁴ Isolates of *M. bovis* were identified by colony morphology, growth, and biochemical characteristics as well as a DNA probe specific for mycobacteria in the *M. tuberculosis* complex (AccuProbe; Gen-Probe Inc., San Diego, CA, USA). Tissues collected for microscopic examination were fixed by immersion in 10% neutral buffered formalin, processed by routine methods to paraffin wax and sectioned ($5 \mu\text{m}$). Sections were stained with hematoxylin and eosin (H/E) for microscopic examination. Adjacent sections were cut from samples containing lesions suggestive of tuberculosis and stained by the Ziehl-Neelsen technique for visualization of acid-fast bacteria.

Tuberculin skin testing

Prior to infection, and 63 and 121 days after infection, all cattle were tested for exposure to *M. bovis* by the comparative cervical test (CCT) for cattle as described in USDA, APHIS, VS guidelines.¹⁵ Briefly, hair was clipped from two sites on the right side of the mid cervical region and the thickness of the skin at each site was measured. One-tenth milliliter (0.4 mg/ml) of *M. avium* purified protein derivative (PPD) (National Veterinary Services Laboratory, Ames, IA) was injected intradermally in the uppermost site and 0.1 ml (1 mg/ml) of *M. bovis* PPD (National Veterinary Services Laboratory) was injected into the lower site. Seventy-two hours after injection, test sites were observed, palpated, and induration measured to the nearest 0.5 mm using calipers. Results were interpreted by plotting measurements of changes in skin thickness at both the *M. bovis* PPD and *M. avium* PPD injection sites on a scattergram developed by USDA for interpretation of the CCT for cattle.¹⁵ The scattergram plots the change in skin thickness at the *M. bovis* PPD injection site against the change in skin thickness measured at the *M. avium* PPD injection site. Although the details of the scattergram are beyond the scope of this paper, in general, animals with large changes in skin thickness at the *M. bovis* PPD injection site with little or no change in skin thickness at the *M. avium* PPD injection site are classified as reactors. Cattle with large changes in skin thickness at the *M. avium* PPD injection site with little or no change at the *M. bovis* PPD injection site as well as cattle with no change at either injection site are classified as negative. Intermediate responses with similar changes in skin thickness at both the *M. bovis* PPD and *M. avium* PPD injection sites are classified as suspects. Results of the CCT were used to classify calves in the present study as negative, suspect, or reactor.

RESULTS

All cattle were classified as negative for exposure to *M. bovis* prior to experimental infection. At both 63 and 121 days after infection, tuberculin skin testing using the comparative cervical skin test classified 19 of 20 calves as reactors and one of 20 as negative for exposure to *M. bovis*. In all groups, regardless of dosage or strain of inoculum, the most commonly affected tissues were the tracheobronchial and mediastinal lymph nodes, and lung (Table 1). With both strains there was dissemination of disease to a larger number of tissues in cattle exposed to higher dosages. Although there appeared to be more sites involved in cattle exposed to the high dose of strain HC2005T, the small number of animals in each group, precludes an accurate assessment of this difference. Lung lesions were most numerous in caudal lung

lobes from all groups (Table 2). Generalized involvement of all lung lobes was more pronounced in those groups receiving the higher dosage of inoculum. This was especially evident in cattle exposed to the high dose of strain HC2005T.

Macroscopic alterations of lymph nodes ranged from variable lymphadenomegaly with no discernible granuloma formation to marked lymphadenomegaly with multiple nodular pale granulomas ranging in size from 0.2 to 2.5 cm. On cut surface, granulomas were gritty and contained central caseonecrotic material (Fig. 2). Grossly visible lung lesions ranged from 0.5 to 5 cm in size, were single or multiple, and involved only one lobe in mild cases or were generalized throughout the lung in severe cases (Fig 3). On cut surface, granulomas within the lung were similar to those seen in lymph nodes. In two of five calves in each of the high-dose groups, multifocal to coalescent, 1–5 cm, irregular, fleshy, pink, nodular masses were present on parietal and costal pleural surfaces (Fig. 4). Two of five calves inoculated with the high dose of *M. bovis* strain HC2005T had gross lesions within the nasal cavity. Such lesions were present on the mid-lateral wall of the nasal cavity and lateral surface of the dorsal and ventral nasal conchae. Lesions consisted of focally extensive, irregular, pink, fleshy, nodular lesions ranging in size from 0.5 to 4 cm (Fig. 5). Similar nasal cavity lesions were not seen in calves infected with *M. bovis* strain 1315.

Microscopic lesions in most tissues consisted of focal, multifocal or coalescent caseonecrotic granulomas composed of central areas of necrosis with some neutrophils, surrounded by epithelioid macrophages, Langhans' type multinucleated giant cells and lymphocytes (Fig. 6). In some cases, the central core of necrotic debris was mineralized. Acid-fast bacteria were seen in low numbers within multinucleated giant cells or extracellularly among cellular debris within the caseonecrotic core. Granulomas often were surrounded by low to moderate numbers of fibroblasts and collagen. Within the lung, many granulomas were found adjacent to or surrounding bronchi or bronchioles (Fig. 7) that contained variable amounts of intraluminal granulomatous to pyogranulomatous infiltrate (Fig. 8). Lesions within the nasal cavity were characterized by marked expansion of the submucosa by coalescent caseonecrotic granulomas. Ulcerations were often seen in overlying epithelial mucosa.

The distribution of tissues from which *M. bovis* was isolated correlated well with lesion distribution (Table 1). However, in the group receiving the low dose of *M. bovis* HC2005T, gross and microscopic lesions were present in the lungs of four of five calves, yet *M. bovis* was not isolated from the lung of any of the calves in this group.

RFLP analysis demonstrated that in each case isolates obtained from mediastinal or tracheobronchial lymph

Table 1 Summary of gross, microscopic and bacteriologic findings from cattle infected by aerosol with a low (1×10^3 CFU) or high dose (1×10^5 CFU) of *M. bovis* strain 1315 or HC2005T.

Tissue	Strains of <i>M. bovis</i>											
	1315						HC2005T					
	Low dose ($n = 5$)			High dose ($n = 5$)			Low dose ($n = 5$)			High dose ($n = 5$)		
	G	M	B	G	M	B	G	M	B	G	M	B
Tonsil	0	0	0	0	0	0	0	0	0	0	0	0
Mandibular LN	0	0	0	2	1	2	0	0	0	3	4	3
Parotid LN	0	0	0	0	0	1	0	0	0	3	3	3
Medial retropharyngeal LN	0	0	0	1	1	1	0	0	0	2	2	2
Prefemoral LN	0	0	0	0	0	0	0	0	0	0	0	0
Tracheobronchial LN	5	5	3	5	5	5	3	4	4	5	5	5
Mediastinal LN	4	4	5	5	5	5	4	4	3	5	5	5
Lung	4	3	2	5	5	4	4	4	0	5	5	5
Mesenteric LN	0	0	0	0	0	0	0	0	0	0	0	0
Hepatic LN	0	0	0	1	1	0	0	0	0	0	0	1
Nasal turbinate	0	0	0	0	0	0	0	0	0	2	2	1
Liver*	3	3		2	2		0	0		3	3	
Kidney*	0	0		0	0		0	0		2	2	

Data represent the total out of five calves in each group that had gross (G) or microscopic (M) lesions or from which *M. bovis* was isolated (B). LN = lymph node

0 = no gross or microscopic lesions, no isolation of *M. bovis*

* Not processed for bacteriologic isolation of *M. bovis*

Table 2 Distribution of tuberculous lesions in lung lobes from cattle infected by aerosol with a low (1×10^3 CFU) or high dose (1×10^5 CFU) of *M. bovis* strain 1315 or HC2005T.

Lung lobe	Strain of <i>M. bovis</i>			
	1315		HC2005T	
	Low dose ($n = 5$)	High dose ($n = 5$)	Low dose ($n = 5$)	High dose ($n = 5$)
Left caudal	4	5	4	5
Right caudal/Middle	4	5	2	5
Left cranial	2	3	0	5
Right cranial	2	5	0	5
Accessory	1	3	0	5

Data represent the total out of 5 calves in each group that had gross and microscopic lesions consistent with tuberculosis or from which *M. bovis* was isolated.

nodes had RFLP patterns identical to that of the inoculum used for that particular animal.

DISCUSSION

Use of the apparatus described in the present study provides a reliable method of aerosol delivery of *M. bovis* to cattle. All but one of the calves in this study developed tuberculous lesions after aerosol exposure to *M. bovis*. *M. bovis* was not isolated from this calf; moreover, no tuberculin skin test reaction, suggestive of exposure to *M. bovis* was seen in this calf, suggesting that the delivery method did not adequately deliver the inoculum in this single case. In all other calves, the distribution of lesions suggests that the aerosol generated by the nebulizer was composed of droplets of diameter less than $5 \mu\text{m}$, which reached the pulmonary alveoli. This was especially

evident in cattle receiving the lower dosage of either strain of *M. bovis*. The droplet nuclei mechanism of infection with *M. tuberculosis* has shown that tiny residues, $<5 \mu\text{m}$, of evaporated droplets (droplet nuclei) can be generated by talking, coughing or even singing. Such nuclei remain airborne for prolonged periods while larger droplets settle out within short distances of their source. Once inhaled, infectious droplet nuclei reach the pulmonary alveoli while droplets $>5 \mu\text{m}$ are removed in the upper respiratory passages.^{16–18} The Equine Aero-mask system is designed for aerosol delivery of pharmaceutical agents to large animals and is easily adapted for the delivery of microbes. Adaptation of aerosol delivery systems currently used for small laboratory animals for use in cattle would likely prove more difficult.

More severe disease, characterized by wider dissemination of disease, with higher inoculum dosages, as seen in

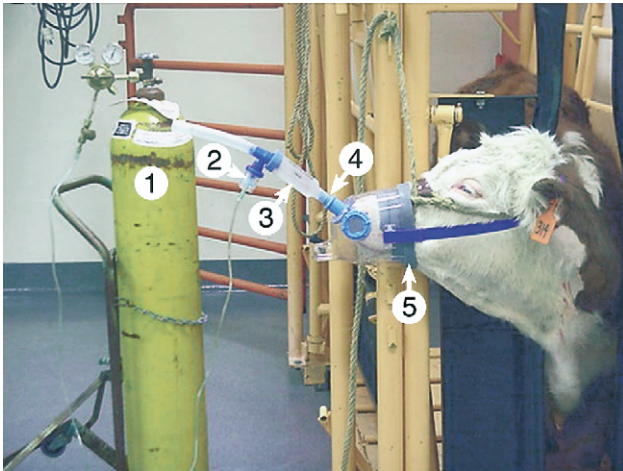


Fig. 1 Aerosol delivery apparatus used to deliver virulent *M. bovis* to cattle. Compressed air (1) is used to aerosolize inoculum using a jet nebulizer (2). Aerosolized inoculum passes into holding chamber (3) until inhaled through one-way valve (4) into mask that is tightly sealed around muzzle using a rubber gasket (5).



Fig. 2 Mediastinal lymph node (top) and tracheobronchial lymph node (bottom) from calf receiving 1×10^5 CFU of *M. bovis* strain HC2005T by aerosol delivery and examined 155 days later. Lymph nodes are enlarged 5–10 times normal and contain coalescent caseonecrotic granulomas.

the present study has also been seen in other animal models of tuberculosis.^{19,20} Unlike animal models of human tuberculosis using mice, rabbits or guinea pigs, the model described herein, uses the animal of interest as the experimental animal, i.e. cattle are used to investigate tuberculosis in cattle. Extrapolation between species that may differ in immune response, lesion development, or susceptibility to infection is therefore, not necessary.

Aerosol delivery of other respiratory pathogens to cattle has been previously reported.²¹ However, the apparatus used consisted of an aerosol chamber into which the calves were placed and nebulized inoculum then filled the chamber, thus exposing the entire animal to inocu-



Fig. 3 Left cranial and caudal lung lobes from calf receiving 1×10^5 CFU of *M. bovis* strain HC2005T by aerosol delivery and examined 155 days later. Note numerous coalescent nodular granulomas of various sizes.



Fig. 4 Internal surface of thorax from calf receiving 1×10^5 CFU of *M. bovis* strain HC2005T by aerosol delivery and examined 155 days later. Note coalescent raised granulomas on pleural surface (arrows).

lum.^{21,22} Such an apparatus limits the size of the animal used, making experimental infection of large cattle difficult. The apparatus used in the present study can be used on cattle ranging from calves to adults and focuses the aerosolized inoculum over the muzzle. Calves rather than cows were used in the present study because of space limitations and animal welfare and housing guidelines.

Numerous abattoir surveys of cattle have described the distribution of lesions in cattle naturally infected with *M. bovis*. Involvement of the lungs and associated lymph nodes ranges from 19% to 80%, while cranial lymph node involvement ranges from 2% to 55%.^{23–28} Data from a large study of 2886 tuberculous cattle revealed that 57% of animals had lesions confined to the lung and associated lymph nodes, 23% had lesions confined to the cranial lymph nodes and 4.7% had lesions in both



Fig. 5 Right side of nasal cavity from calf receiving 1×10^5 CFU of *M. bovis* strain HC2005T by aerosol delivery and examined 155 days later. Note multiple raised granulomas in mid-nasal cavity (nasal conchae removed).

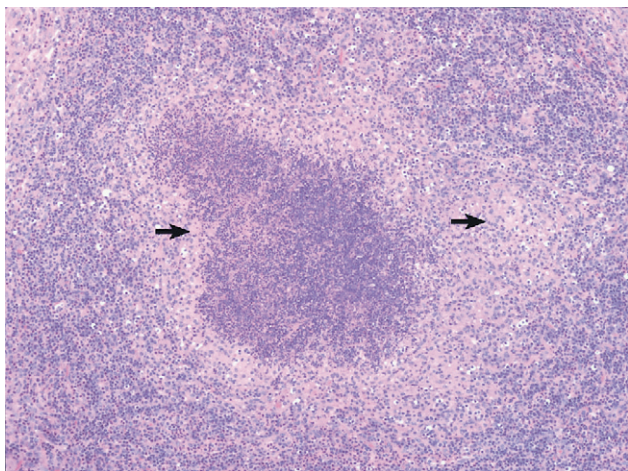


Fig. 6 Photomicrograph of section of mediastinal lymph node from a calf receiving 1×10^3 CFU of *M. bovis* strain 1315 by aerosol delivery and examined 155 days later. Note typical granuloma with central necrotic debris and neutrophils surrounded by epithelioid macrophages and Langhans' type giant cells (arrows). H/E. 100 \times magnification.

cranial and thoracic sites.⁷ The distribution of lesions may vary depending on age as the distribution of lesions among young heifers is 20% cranial lymph nodes, 22% lungs and associated lymph nodes, and 8% both cranial and thoracic sites while distribution among mature cows is 9% cranial lymph nodes, 37% lungs and associated lymph nodes and 9% both cranial and thoracic sites.²⁸ Breed differences may also be apparent as one Australian study revealed cranial lymph node involvement in 7% of the cases in beef cattle, while 55% of dairy cattle had cranial lymph node lesions.²⁹ In the present study, involvement of the cranial lymph nodes was limited to cattle receiving higher dosages of inoculum. Therefore,

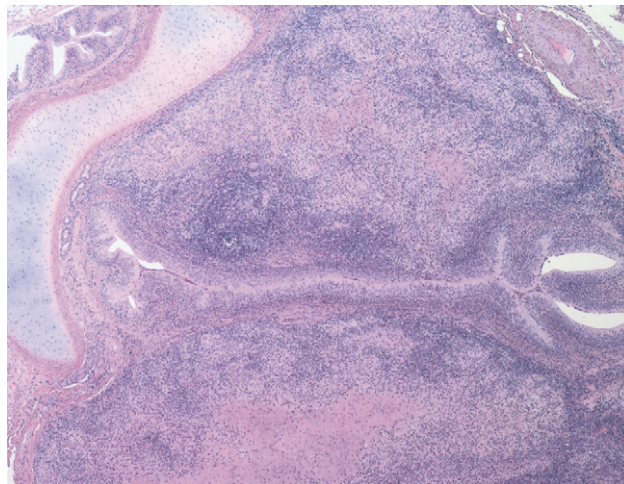


Fig. 7 Photomicrograph of section of lung from a calf receiving 1×10^3 CFU of *M. bovis* strain HC2005T by aerosol delivery and examined 155 days later. Lumen of airway is compressed by expansive granulomas surrounding bronchus. H/E. 40 \times magnification.

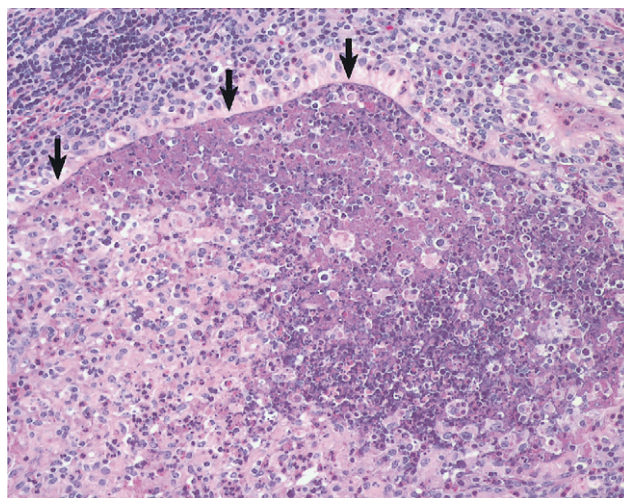


Fig. 8 Photomicrograph of a section of lung from a calf receiving 1×10^5 CFU of *M. bovis* strain 1315 by aerosol delivery and examined 155 days later. Airway lumen is filled with exudate composed of macrophages and neutrophils. Bronchiolar epithelium (arrows). H/E. 200 \times magnification.

the distribution of lesions seen in the present study is similar to that seen in natural *M. bovis* infection and demonstrates that the wide range of lesion distribution seen in naturally infected cattle may be due to dosage of exposure as well as age and breed of the animal.

The involvement of the cranial lymph nodes may result from either oral or aerosol exposure to *M. bovis*. Specifically, the medial retropharyngeal lymph nodes receive lymphatic drainage from the posterior pharynx including the tonsils.³⁰ Microbes and other antigens

deposited on the mucosal surface of the posterior pharynx, regardless of route of delivery, are likely to be processed by the tonsil and medial retropharyngeal lymph node. Based on abattoir studies and the low incidence of alimentary tract lesions in tuberculous cattle, it has been estimated that approximately 90% of pharyngeal lymph node lesions result from aerosol rather than oral exposure to *M. bovis*.^{28,29}

A predilection for lesion development in the caudal lung lobes, similar to that seen in the present study, has previously been reported in cattle^{23,31} and white-tailed deer.³² Moreover, approximately half of those lesions reported are in the distal third of the caudal lobes.³³ The reason for such a predilection is unclear. Gross lesions of the upper respiratory tract (nasal mucosa, turbinates, septum and nasopharynx) similar to those reported in the present study, have been seen in cattle as early as 17 days after intranasal inoculation.⁹ Such early lesions are likely the result of inoculation route. In the present study, it is unclear if nasal mucosal lesions were the direct result of inoculation route or rather exhaled respiratory droplets containing *M. bovis* generated from lung lesions.

The method of aerosol delivery described above represents a reliable method of experimental infection of cattle with *M. bovis*. This method results in a distribution of lesions similar to that described in naturally infected cattle and could serve as a useful challenge model to evaluate disease pathogenesis, immune response, mycobacterial shedding or vaccine efficacy. Likewise, it would be useful for other respiratory pathogens of cattle where aerosol exposure is critical.

ACKNOWLEDGMENTS

The authors thank Rebecca Lyon, Jody Mentale, Theresa Rahner, Nicole Heley-Lehman, and Ryan Cook for technical assistance and Larry Wright, Nate Horman, Don Robinson, Wayne Varland, Doug Ewing, and Norm Lyon, for animal care.

REFERENCES

1. Dannenburg A M. Rabbit model of tuberculosis. In: Bloom B R, ed. *Tuberculosis: Pathogenesis, Protection and Control*. Washington, DC: ASM Press, 1994: pp 149–156.
2. McMurray D N. Guinea pig model of tuberculosis. In: Bloom B R, ed. *Tuberculosis: Pathogenesis, Protection and Control*. Washington, DC: ASM Press, 1994: pp 135–147.
3. Orme I M, Collins F M. Mouse model of tuberculosis. In: Bloom B R, ed. *Tuberculosis: Pathogenesis, Protection and Control*. Washington, DC: ASM Press, 1994: pp 113–134.
4. Palmer M V, Whipple D L, Olsen S C. Development of a model of natural infection with *Mycobacterium bovis* in white-tailed deer. *J Wild Dis* 1999; 35: 450–457.
5. Buchan G S, Griffin J F T. Tuberculosis in domesticated deer (*Cervus elaphus*): a large animal model for human tuberculosis. *J Comp Pathol* 1990; 103: 11–22.
6. Grange J M, Yates M D. Zoonotic aspects of *Mycobacterium bovis* infection. *Vet Microbiol* 1994; 40: 137–151.
7. Neill S D, Pollock J M, Bryson D B, Hanna J. Pathogenesis of *Mycobacterium bovis* infection in cattle. *Vet Microbiol* 1994; 40: 41–52.
8. Cassidy J P, Bryson D G, Pollock J M, Evans R T, Forster F, Neill S D. Lesions in cattle exposed to *Mycobacterium bovis*-inoculated cattle. *J Comp Pathol* 1999; 121: 321–327.
9. Cassidy J P, Bryson D G, Pollock J M, Evans R T, Forster F, Neill S D. Early lesion formation in cattle experimentally infected with *Mycobacterium bovis*. *J Comp Pathol* 1998; 119: 27–44.
10. Buddle B M, Aldwell F E, Pfeffer A, de Lisle G W, Corner L A. Experimental *Mycobacterium bovis* infection of cattle: effect of dose of *M. bovis* and pregnancy on immune responses and distribution of lesions. *NZ Vet J* 1994; 42: 167–172.
11. Palmer M V, Whipple D L, Rhyan J C, Bolin C A, Saari D A. Granuloma development in cattle after intratracheal inoculation with *Mycobacterium bovis*. *Am J Vet Res* 1999; 60: 310–315.
12. Bolin C A, Whipple D L, Khanna K V, Risdahl J M, Peterson P K, Molitor T W. Infection of swine with *Mycobacterium bovis* as a model of human tuberculosis. *J Infect Dis* 1997; 176: 1559–1566.
13. Whipple D L, Clarke P R, Jarnagin J L, Payeur J B. Restriction fragment length polymorphism analysis of *Mycobacterium bovis* isolates from captive and free-ranging animals. *J Vet Diagn Invest* 1997; 9: 381–386.
14. Hess D, Fisher D, Williams P, Pooler S, Kacmarek R M. Medication nebulizer performance: effect of diluent volume, nebulizer flow and nebulizer brand. *Chest* 1996; 110: 498–505.
15. United States Department of Agriculture. Animal and Plant Health Inspection Service, Veterinary Services. Bovine Tuberculosis eradication uniform methods and rules. US Government Printing Office, Washington, DC, 21 pp.
16. Wells W F, Ratcliffe H L, Crumb C. On the mechanisms of droplet nuclei infection. II. Quantitative experimental air-borne tuberculosis in rabbits. *Am J Hyg* 1948; 47: 11–28.
17. Loudon G L, Roberts R M. Droplet expulsion from the respiratory tract. *Am Rev Resp Dis* 1967; 95: 435–442.
18. Loudon R G, Roberts R M. Singing and the dissemination of tuberculosis. *Am Rev Resp Dis* 1968; 98: 297–300.
19. Chambers M A, Williams A, Gavier-Widen D et al. A guinea pig model of low-dose *Mycobacterium bovis* aerogenic infection. *Vet Microbiol* 2001; 80: 213–226.
20. Converse P J, Dannenburg A M, Estep J E et al. Cavitory tuberculosis produced in rabbits by aerosolized virulent tubercle bacilli. *Infect Immun* 1996; 64: 4776–4787.
21. Tegtmeier C, Angen Ø, Grell S N, Riber U, Friis N F. Aerosol challenge of calves with *Haemophilus somnus* and *Mycoplasma dispar*. *Vet Microbiol* 2000; 72: 229–239.
22. Jacobsen M J, Nielsen J P, Nielsen R. Comparison of virulence of different *Actinobacillus pleuropneumoniae* serotypes and biotypes using an aerosol infection model. *Vet Microbiol* 1996; 49: 159–168.
23. McKay W M. A clinical study of bovine tuberculosis in Banffshire: the pathological lesions. *Br Vet J* 1959; 115: 324–329.
24. Milian-Suazo F, Salman M D, Ramirez C, Payeur J B, Rhyan J C, Santillan M. Identification of tuberculosis in cattle slaughtered in Mexico. *Am J Vet Res* 2000; 61: 86–89.
25. Whipple D L, Bolin C A, Miller J M. Distribution of lesions in cattle infected with *Mycobacterium bovis*. *J Vet Diagn Invest* 1996; 8: 351–354.
26. Lepper A W D, Pearson C W. The route of infection in tuberculosis of beef cattle. *Aust Vet J* 1973; 49: 266–267.
27. Claxton P D, Eamens G J, Mylrea P J. Laboratory diagnosis of bovine tuberculosis. *Aust Vet J* 1979; 55: 514–520.

28. Francis J. Route of infection in tuberculosis. Aust Vet J 1972; 48: 578.
29. Stamp J T, Wilson A. Some aspects of the pathogenesis of bovine tuberculosis, based on abattoir returns. Vet Rec 1946; 58: 11–15.
30. Saar L L, Getty R. Ruminant lymphatic system. In: Getty R, ed. The Anatomy of the Domestic Animals. Philadelphia: W B Saunders, 1975: p 1027.
31. Stamp J T. Bovine pulmonary tuberculosis. J Comp Pathol 1948; 58: 9–23.
32. Palmer M V, Waters W R, Whipple D L. Lesion development in white-tailed deer (*Odocoileus virginianus*) experimentally infected with *Mycobacterium bovis*. Vet Pathol 2002; 39: 334–340.
33. McIlroy S G, Neill S D, McCracken R M. Pulmonary lesions and *Mycobacterium bovis* excretion from the respiratory tract of tuberculin reacting cattle. Vet Rec 1986; 118: 718–721.